Exhibit 24

In vitro Removal of Stain with Dentifrices

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An in vitro *model has been developed for the purpose of evaluating the cleaning ability of dentifrices. A stained film is slowly deposited on enamel sections and subsequently removed using a mechanical brushing procedure. Results from this test procedure compare favorably with the results of controlled, clinical cleaning studies.*

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Introduction.

Since the initial report by Manly,[1] the presence of a tenacious, recurrent, structureless film or pellicle on exposed tooth surfaces has been described by a number of investigators.[2-8] The removal, or control, of this pellicle accumulation is considered important, since the material is frequently stained and is cosmetically undesirable. It has been repeatedly demonstrated that controlling the accumulation of this pellicle is dependent upon the periodic brushing of the teeth with an abrasive material.[2,9-14]

A variety of clinical procedures has been reported for assessing the ability of dentifrice abrasives to prevent the accumulation of stained pellicle.[10,12-18] In general, these procedures have involved clinical assessments of either the removal of stained pellicle or the prevention of stained pellicle accumulation. However, the use of such expensive and time-consuming procedures for the screening and development of more efficient abrasive systems is impractical.

Attempts to develop suitable laboratory procedures for this purpose have generally resulted in limited success. The major obstacle has been in the identification of a suitable substrate where the ease of removal using controlled tooth-brushing conditions parallels that of stained pellicle in clinical situations. Efforts to stain enamel specimens with tobacco tars, tobacco extracts, or a number of organic and inorganic stains have not been successful due to the lack of tenacity of the materials to intact enamel.[19] Cobalt sulfide precipitated on porcelain has been used,[19] but no attempt has been made to correlate the results with clinical observations. A lacquer film on polyester blocks has also been suggested;[20,21] however, the use of organic films of this type is restricted to studies of simple abrasive systems, since some dentifrice components (*e.g.*, flavoring agents) may act as solvents and yield misleading results.

The modest success of these latter laboratory endeavors emphasized the need to identify a more appropriate substrate. A recent report[22] involved the use of prosthetic appliances containing enamel specimens which could be periodically removed and subjected to controlled tooth-brushing procedures. While the latter approach has considerable appeal (since the substrate is most appropriate), the practical limitations of the method are obvious. In view of this situation, efforts were made to identify a practical laboratory procedure capable of predicting, with, at least, moderate success, the ability of abrasives and complete dentifrices (*i.e.*, with surfactants, flavoring agents, *etc.*) to remove stained pellicle in clinical situations. This report summarizes the method which evolved from these efforts.

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Materials and methods.

Bovine permanent central incisors were cut to obtain labial enamel specimens approximately 10 mm^2. The enamel specimens were embedded in an autopolymerizing methacrylate resin in such a manner that only the enamel surfaces were exposed. The specimens were embedded with the aid of a mold which was the appropriate size (12 mm^2) so as to facilitate the positioning of the specimens in the heads of a V-8 cross-brushing machine.[23] The enamel surfaces were smoothed and polished on a lapidary wheel utilizing 100 grit, followed by 600 grit, wet sandpaper under a constant flow of water. The specimens were lightly etched to expedite stain accumulation and adherence. This etching procedure consisted of a 60-second immersion in 0.12 N (1%) hydrochloric acid, followed by a 30-second immersion in a super-saturated solution of sodium carbonate; a final etch was performed with 1% phytic acid for 60 s. The specimens were then rinsed in deionized water and were attached to the staining apparatus.

The staining apparatus was constructed to provide alternate immersion into the staining broth and air drying of the specimens. The apparatus consisted of a platform which supported a Teflon rod connected to a 2-rpm motor. The Teflon rod contained holes through which the specimens were attached by means of pieces of nichrome wire. Beneath the rod was a removable trough with a 500 ml capacity. The trough contained the staining broth, which consisted of 2.7 g of finely-ground instant coffee, 2.7 g of finely-ground instant tea, and 2.0 g of finely-ground gastric mucin* dissolved into 800 ml of sterilized trypticase soy broth. Twenty-six ml of a 24-hour Sarcina lutea turtox culture were also added to the staining broth. The apparatus, with the enamel specimens attached and the staining broth in place, was then placed in an incubator (37°C) with the specimens rotating continuously through the staining broth and air. The staining broth was replaced twice daily for four consecutive d. With each broth change, the trough and specimens were rinsed with deionized water to remove any loose deposits. After the four-day staining period, a darkly-stained film or coating was apparent on the enamel surfaces. The specimens were removed from the staining apparatus, rinsed well, allowed to air dry for about ten min, and then refrigerated until used.

The amount of *in vitro* stain was subjectively graded with the aid of a binocular microscope at a magnification of 25X. For this purpose, each specimen was arbitrarily divided into six equal sub-areas, and the stain on each area was assessed using scores ranging from zero to five, indicative of the amount of coverage of stain. The arbitrary numerical scale and visual grading criteria were as follows: zero, no visible stain; one, light stain covering up to 75% of the area; two, light stain covering from 75 to 100% of the area or medium stain covering up to 75% of the area; three, medium stain covering from 75 to 100% of the area or heavy stain covering up to 25% of the area; four, heavy stain covering from 25 to 50% of the area; and five, heavy stain covering from 50 to 100% of the area. The terms light,

*Gastric mucin N.N.R., Nutritional Biochemicals Corp., Cleveland, OH 44128

medium, and heavy were used primarily to characterize the color intensity of the stain; although the thickness of the stain was never measured, the stain also appeared to increase in thickness along with increasing intensity of pigmentation. The numerical values for the six areas were totaled to obtain the baseline score for each specimen. The maximum baseline score was 30; however, only specimens with baseline scores of 22-28 were used for these studies. This range of baseline stain scores was selected on the basis of prior studies, which indicated that greater variability in stain removal was observed with both greater (*i.e.*, scores of 28-30) and lesser (*i.e.*, scores of <22) amounts of stain accumulation.

Using the baseline scores, the specimens were divided and balanced into the desired number of groups of eight specimens each, with each group having equivalent average baseline scores. The specimens were then mounted on a V-8 mechanical cross-brushing machine[23] equipped with soft nylon-filament toothbrushes† adjusted to 150 g tension upon the enamel surface. The dentifrices were tested as a slurry consisting of 25 g of dentifrice mixed with 40 ml of deionized water, and the specimens were brushed for 800 double strokes. To minimize mechanical variables, only one enamel specimen from each group was brushed on any given brushing head, and each test product was included in each run. Following brushing, the specimens were rinsed, blotted dry, and graded again. The difference between pre- and post-test scores was considered to represent the ability of the test material to remove the stain.

The early studies included a slurry of a standard lot of calcium pyrophosphate[24] as an internal reference in each test, and this material was assigned an arbitrary cleaning value of 100. The cleaning scores for all test materials were then expressed as a ratio:

$$\frac{\text{Mean decrement for test material}}{\text{Mean decrement for reference material}} \times 100 = \text{cleaning ratio}$$

For the present stain studies, a standard lot of a commercial dentifrice‡ which contained a high beta-phase calcium pyrophosphate was used as the reference material; this formulation was found to have a cleaning ratio of 130 relative to the standard calcium pyrophosphate and was assigned this value in all subsequent tests.

Results.

The *in vitro* test procedure was used to evaluate the cleaning potential of three experimental dentifrices involved in a collaborative clinical cleaning methodology study conducted by the ADA Dentifrices Function Committee.[25] This clinical trial involved an evaluation of three dentifrices prepared especially for the study and known to differ significantly in abrasivity to dentin. The clinical study was also designed to assess dentifrice performance both with regard to the removal of existing stain and prevention of stain accumulation.

The results observed with these dentifrices are shown in Table 1. The dentin abrasivity values obtained with the conventional radiotracer procedure[24] indicate that the dentifrices differed markedly, with abrasivity ratio values ranging from 85 to 245 relative to a value of 100 for the calcium pyrophosphate reference material. The mean baseline stain scores were quite comparable for each group. The average stain scores obtained after the toothbrushing procedure differed significantly for the various dentifrices and ranged from 9.5 to 18.6 for the dentifrices having higher and lower abrasivity, respectively. The latter stain scores and the corresponding stain decrements indicate that about 30-64% of the stain was removed by the dentifrices.

The *in vitro* and clinical cleaning ratios for these dentifrices are presented in Table 2. Since the clinical study did not contain the reference systems used in the *in vitro* model, the moderately abrasive dentifrice was used to calculate the cleaning ratios for both test procedures. The clinical cleaning ratios ranged from 80 to 128 for the stain removal procedures, while cleaning ratios for the laboratory test scores ranged from 59 to 128. Although the numerical values varied between models, it is significant that the laboratory test ranked the products in the same order.

In order to further investigate the ability of this *in vitro*-stained pellicle model to predict clinical cleaning ability, tests were performed using a total of 22 experimental dentifrices which had been evaluated in 12 separate clinical trials. All the clinical studies were conducted independent of the present study, and the test products were simply used in this investigation to permit attempts to correlate the *in vitro* data with clinical results. The clinical trials were conventional cross-over pellicle stain studies typically involving two to four products and about 75-100 patients; the clinical test procedures were essentially those described by Sturzenberger[25] designed to investigate the rate of stained pellicle accumulation on previously cleaned tooth surfaces. Clinical stain gradings were made with the aid of a 15X binocular microscope.

The Fig. summarizes the results of this study and indicates a general direct relationship between the cleaning ratios observed in the *in vitro* and clinical tests. A Pearson correlation coefficient was calculated between the results of the two methods, and an r value of +0.85 was obtained, indicative of a high degree of correlation between the two sets of data.

†Oral B-40, Oral B Co., Inc., Fairfield, NJ 07006
‡Gleem, Procter & Gamble Co., Cincinnati, OH

TABLE 1
IN VITRO STAIN REMOVAL DATA ON ADA CLINICAL PRODUCTS

Dentifrice Abrasivity	Dentin Abrasivity Index[25]	Baseline Stain Score	Post-test Stain Score	Stain Decrement	Percent Stain Removal
Higher	245 ± 6*	26.4 ± 0.8 **	9.5 ± 1.9	16.9 ± 1.7	64.0
Intermediate	165 ± 3	26.7 ± 0.8	13.4 ± 1.7	13.3 ± 1.4	49.8
Lower	85 ± 2	26.5 ± 0.8	18.6 ± 1.8	7.9 ± 1.3	29.8

*Mean ± standard error of the mean.
**Values within brackets do not differ significantly ($P < 0.05$) according to Newman-Keul's analysis.

TABLE 2
IN VITRO DATA ON ADA CLINICAL PRODUCTS

Dentifrice Abrasivity	Dentin Abrasivity Index[25]	Clinical Cleaning Ratio		*In vitro* Cleaning Ratio
		Removal	Prevention	
Higher	245 ± 6	128	114	128
Intermediate	165 ± 3	100	100	100
Lower	85 ± 2	80	85	59

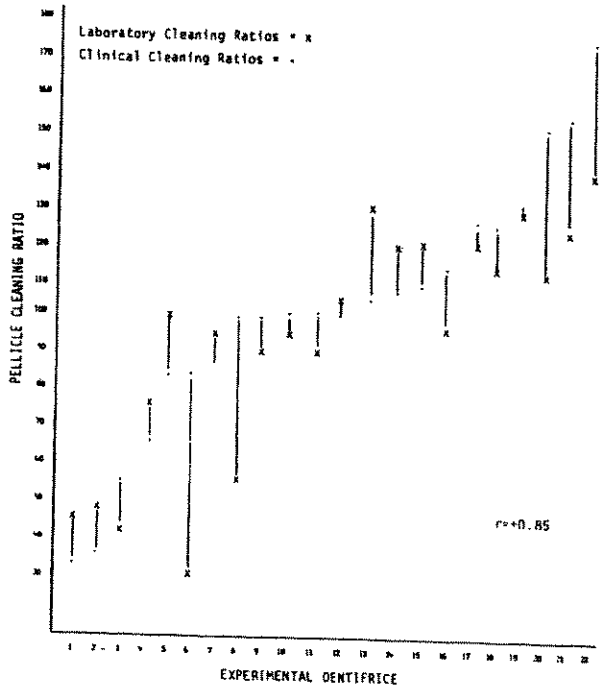

Fig. – Comparison of clinical and *in vitro* cleaning ratios for 22 different dentifrices.

Discussion.

As noted earlier, the reported procedure for developing an *in vitro* stained substrate is that which evolved after numerous attempts and failures using other types of systems. Among the systems which were explored were a variety of proteins, such as zein and casein, and various forms of tobacco (tars, smoke, chewing tobacco, *etc.*). None of these approaches resulted in adequate amounts of stain, and the stain which did form was easily removed. The use of saliva, as a source of glycoprotein, appeared to be suitable in terms of stain formation, but was considered impractical due to the quantities required daily. The use of a slight etch on the enamel greatly enhanced the rate of stain accumulation with unetched specimens requiring four to six wk to develop comparable stain.

The controlled toothbrushing conditions used with this staining procedure were also identified through a series of tests comparing three dentifrices known to differ in cleaning ability from prior clinical stain studies. The laboratory studies were designed particularly to identify the appropriate tension or load on the toothbrushes and the number of toothbrushing strokes. A soft nylon toothbrush having a flat trim was selected on the basis of the common use of this type of toothbrush; no studies comparing different toothbrushes were conducted. The composition of the dentifrice slurries was selected from previous investigations of abrasivity.[24]

As illustrated in the Fig., this laboratory test procedure appears to be capable of predicting the ability of a dentifrice to remove stained pellicle under clinical usage conditions. While numerical values for laboratory cleaning ratios vary appreciably from corresponding clinical values in some instances, the laboratory procedure appears to provide an adequate degree of correlation ($r = +0.85$) to be useful. However, it should be stressed that laboratory procedures such as this are not intended to replace clinical evaluations, but, rather, should be used to screen abrasives and formulations, thereby identifying those having the greatest potential for success in clinical studies.

Conclusions.

An *in vitro* procedure has been developed for estimating the cleaning potential of dentifrices. This method relies on the formation of a stained film on enamel specimens; the staining procedure involves intermittent bathing of the enamel in a mixture consisting of mucin, coffee, tea, microbiological media, and a chromogenic microorganism. The ability of dentifrices or dentifrice cleaning and polishing agents to remove this stain using controlled toothbrushing conditions is then determined. The *in vitro* procedure provided cleaning ratios which correlated well ($r = 0.85$) with the clinical results observed with the same products. This procedure thus appears useful as a means of screening and identifying dentifrice cleaning and polishing agents, as well as dentifrice formulations worthy of clinical evaluation.

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Participate: 27th Annual Edward H. Hatton Awards Competition for Junior Investigators.

Entries are being solicited for this competition, to be held at the IADR's 61st General Session (August 1-3, 1983, Sydney, Australia).

As always, entries are judged in two separate categories: Pre-doctoral and Post-doctoral. Pre-doctoral entrants are defined as those who do not possess a license or degree to practice dentistry, or a doctorate in a scientific subject. Post-doctoral entrants are defined as those who hold a dental license or degree, or a doctorate in a scientific subject.

Seven copies of the following must be submitted with each entry:

(a) the manuscript of the competing paper, complete with illustrations and bibliography prepared according to "Instructions to Authors" (*Journal of Dental Research* 61:957, 1982);

(b) biographical sketch of the author;

(c) recommendation from the author's sponsor; and

(d) abstract of the paper submitted in standard IADR abstract format.

Send entries to Professor Declan J. Anderson, Chairman, Hatton Committee, Department of Physiology, Medical School, University Walk, Bristol BS8 1TD, England. Entries must be received by **January 31, 1983.**

Brochures and flyers with more complete details were mailed worldwide in August. Copies may also be obtained from the Central Office. **NOTE:** The IADR Board of Directors has approved a policy change whereby winners of Divisional awards will be eligible to compete for Hatton Awards. This will allow for publication of their abstracts in the IADR *Program & Abstracts*, even after having been published in Divisional programs. Entrants should also be reminded that, if they win, their papers will be printed in the JDR.

SPECIAL NOTE: Travel assistance will be available, in the amount of at least $500 per person, to be awarded on the basis of merit, need, and geographical distribution, as determined by the Hatton Committee. This money is to be used only for travel costs.